

PYROPHOSPHOROLYSIS AND INCORPORATION OF NUCLEOTIDE  
METHOD FOR NUCLEIC ACID DETECTION

Description

Cross-Reference to Related Applications

This application is a continuation-in-part of allowed U.S. Serial No. 09/406,064 filed September 27, 1999, which is a continuation-in-part of U.S. Serial No. 09/358,972, filed on July 21, 1999, now U.S. Patent No. 6,235,480 issued May 22, 2001, which is a continuation-in-part of U.S. Serial No. 09/252,436, filed on February 18, 1999, now U.S. Patent No. 6,159,693, issued December 12, 2000, which is a continuation-in-part of allowed U.S. Serial No. 09/042,287, filed March 13, 1998, all of which are incorporated herein by reference.

Technical Field

The invention relates to nucleic acid detection. More specifically, the invention relates to the determination of the presence or absence of targeted, predetermined nucleic acid sequences in nucleic acid target/probe hybrids, and the various applications of their detection.

Background of the Invention

Methods to detect specific nucleic acids provide a foundation upon which the large and rapidly growing field of molecular biology is built. There is constant need for alternative methods and products. The reasons for selecting one method over another are varied, and include a desire to avoid radioactive materials, the lack of a license to use a technique, the cost or availability of reagents or equipment, the desire to minimize the time spent or

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the number of steps, the accuracy or sensitivity for a certain application, the ease of analysis, the need to detect multiple nucleic acids in one sample, or the ability to automate the process.

The detection of specific nucleic acids is often a portion of a process rather than an end in itself. There are many applications of the detection of nucleic acids in the art, and new applications are always being developed. The ability to detect and quantify nucleic acids is useful in detecting microorganisms, viruses and biological molecules, and thus affects many fields, including human and veterinary medicine, food processing and environmental testing. Additionally, the detection and/or quantification of specific biomolecules from biological samples (e.g. tissue, sputum, urine, blood, semen, saliva) has applications in forensic science, such as the identification and exclusion of criminal suspects and paternity testing as well as medical diagnostics.

Some general methods to detect nucleic acid hybrids are not dependent upon a *priori* knowledge of the nucleic acid sequence. Duplex DNA can be detected using intercalating dyes such as ethidium bromide. Such dyes are also used to detect hybrid formation.

Several hybridization methods to detect nucleic acids are dependent upon knowledge of the nucleic acid sequence. Many known nucleic acid detection techniques depend upon specific nucleic acid hybridization in which an oligonucleotide probe is hybridized or annealed to nucleic acid in the sample or on a blot, and the hybridized probes are detected.

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$$T_{\text{eff}} = \frac{\sum_i T_i^2}{n} - \left( \frac{\sum_i T_i}{n} \right)^2$$
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Fluorescence techniques are also known for the detection of nucleic acid hybrids, U.S. Patent No. 5,691,146 describes the use of fluorescent hybridization probes that are fluorescence-quenched unless they are hybridized to the target nucleic acid sequence. U.S. Patent No. 5,723,591 describes fluorescent hybridization probes that are fluorescence-quenched until hybridized to the target nucleic acid sequence, or until the probe is digested. Such techniques provide information about hybridization, and are of varying degrees of usefulness for the determination of single base variances in sequences. Some fluorescence techniques involve digestion of a nucleic acid hybrid in a 5'→3' direction to release a fluorescent chemical entity from proximity to a fluorescence quencher, for example, TaqMan® (Perkin Elmer; U.S. Patent No. 5,691,146 and No. 5,876,930).

Enzymes having template-specific polymerase activity for which some 3'→5' depolymerization

activity has been reported include *E. coli* DNA Polymerase (Deutscher and Kornberg, *J. Biol. Chem.*, **244**(11):3019-28 (1969)), T7 DNA Polymerase (Wong et al., *Biochemistry* **30**:526-37 (1991); Tabor and Richardson, *J. Biol. Chem.* **265**: 8322-28 (1990)), *E. coli* RNA polymerase (Rozovskaya et al., *Biochem. J.* **224**:645-50 (1994)), AMV and RLV reverse transcriptases (Srivastava and Modak, *J. Biol. Chem.* **255**: 2000-4 (1980)), and HIV reverse transcriptase (Zinnen et al., *J. Biol. Chem.* **269**:24195-202 (1994)). A template-dependent polymerase for which 3' to 5' exonuclease activity has been reported on a mismatched end of a DNA hybrid is phage 29 DNA polymerase (de Vega, M. et al. *EMBO J.*, **15**:1182-1192, 1996).

A variety of methodologies currently exist for the detection of single nucleotide polymorphisms (SNPs) that are present in genomic DNA. SNPs are DNA point mutations or insertions/deletions that are present at measurable frequencies in the population. SNPs are the most common variations in the genome. SNPs occur at defined positions within genomes and can be used for gene mapping, defining population structure, and performing functional studies. SNPs are useful as markers because many known genetic diseases are caused by point mutations and insertions/deletions. Some SNPs are useful as markers of other disease genes because they are known to cosegregate.

In rare cases where an SNP alters a fortuitous restriction enzyme recognition sequence, differential sensitivity of the amplified DNA to cleavage can be used for SNP detection. This technique requires that an appropriate restriction enzyme site be present or introduced in the

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Single strand conformational polymorphism (SSCP) is a second technique that can detect SNPs present in an amplified DNA segment (Hayashi, K. *Genetic Analysis: Techniques and Applications* 9:73-79, 1992). In this method, the double stranded amplified product is denatured and then both strands are allowed to reanneal during electrophoresis in non-denaturing polyacrylamide gels. The separated strands assume a specific folded conformation based on intramolecular base pairing. The electrophoretic properties of each strand are dependent on the folded conformation. The presence of single nucleotide changes in the sequence can cause a detectable change in the conformation and electrophoretic migration of an amplified sample relative to wild type samples, allowing SNPs to be identified. In addition to the limited throughput possible by gel-based techniques, the design and interpretation of SSCP based experiments can be difficult. Multiplex analysis of several samples in the same SSCP reaction is extremely challenging. The sensitivity required in mutation detection and analysis has led most investigators to use radioactively labeled PCR products for this technique.

In the amplification refractory mutation system (ARMS, also known as allele-specific PCR or ASPCR), two amplification reactions are used to determine if a SNP is present in a DNA sample (Newton et al. *Nucl Acids Res* 17:2503, 1989; Wu et al. *PNAS* 86:2757, 1989). Both amplification reactions contain a common primer for the target of interest. The first reaction contains a second primer specific for the wild type product which will give rise to a PCR product if the wild type gene is present in the sample. The second PCR reaction contains a primer that has a single nucleotide change at or near the 3' end that represents the base change that is present in the mutated form of the DNA. The second primer, in conjunction with the common primer, will only function in PCR if genomic DNA that contains the mutated form of genomic DNA is present. This technique requires duplicate amplification reactions to be performed and analyzed by gel electrophoresis to ascertain if a mutated form of a gene is present. In addition, the data must be manually interpreted.

Single base extension (GBA®) is a technique that allows the detection of SNPs by hybridizing a single strand DNA probe to a captured DNA target (Nikiforov, T. et al. *Nucl Acids Res* 22:4167-4175). Once hybridized, the single strand probe is extended by a single base with labeled dideoxynucleotides. The labeled, extended products are then detected using colorimetric or fluorescent methodologies.

A variety of technologies related to real-time (or kinetic) PCR have been adapted to perform SNP detection. Many of these systems are platform based, and require specialized equipment, complicated primer design, and expensive supporting materials for SNP detection. In contrast, the process of this

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invention has been designed as a modular technology that can use a variety of instruments that are suited to the throughput needs of the end-user. In addition, the coupling of an automatable format with standard oligonucleotide chemistry and well-established enzymology provides a flexible and open system architecture. Alternative analytical detection methods, such as mass spectroscopy, HPLC, and fluorescence detection methods can also be used in the process of this invention, providing additional assay flexibility.

SNP detection using real-time amplification relies on the ability to detect amplified segments of nucleic acid as they are generated during the amplification reaction. Three basic real-time SNP detection methodologies exist: (i) increased fluorescence of double strand DNA specific dye binding, (ii) decreased quenching of fluorescence during amplification, and (iii) increased fluorescence energy transfer during amplification (Wittwer, C. et al. *Biotechniques* **22**:130-138, 1997). All of these techniques are non-gel based and each strategy will be briefly discussed.

A variety of dyes are known to exhibit increased fluorescence in response to binding double stranded DNA. This property is utilized in conjunction with the amplification refractory mutation system described above to detect the presence of SNP. Production of wild type or mutation containing PCR products are continuously monitored by the increased fluorescence of dyes such as ethidium bromide or Syber Green as they bind to the accumulating PCR product. Note that dye binding is not selective for the sequence of the PCR product,

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and high non-specific background can give rise to false signals with this technique.

A second detection technology for real-time PCR, known generally as exonuclease primers (TaqMan® probes), utilizes the 5' exonuclease activity of thermostable polymerases such as *Taq* to cleave dual-labeled probes present in the amplification reaction (Wittwer, C. et al. *Biotechniques* **22**:130-138, 1997; Holland, P et al *PNAS* **88**:7276-7280, 1991). While complementary to the PCR product, the probes used in this assay are distinct from the PCR primer and are dually-labeled with both a molecule capable of fluorescence and a molecule capable of quenching fluorescence. When the probes are intact, intramolecular quenching of the fluorescent signal within the DNA probe leads to little signal. When the fluorescent molecule is liberated by the exonuclease activity of *Taq* during amplification, the quenching is greatly reduced leading to increased fluorescent signal.

An additional form of real-time PCR also capitalizes on the intramolecular quenching of a fluorescent molecule by use of a tethered quenching moiety. The molecular beacon technology utilizes hairpin-shaped molecules with an internally-quenched fluorophore whose fluorescence is restored by binding to a DNA target of interest (Kramer, R. et al. *Nat. Biotechnol.* **14**:303-308, 1996). Increased binding of the molecular beacon probe to the accumulating PCR product can be used to specifically detect SNPs present in genomic DNA.

A final, general fluorescent detection strategy used for detection of SNP in real time utilizes synthetic DNA segments known as hybridization probes in conjunction with a process

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known as fluorescence resonance energy transfer (FRET) (Wittwer, C. et al. *Biotechniques* 22:130-138, 1997; Bernard, P. et al. *Am. J. Pathol.* **153**:1055-1061, 1998). This technique relies on the independent binding of labeled DNA probes on the target sequence. The close approximation of the two probes on the target sequence increases resonance energy transfer from one probe to the other, leading to a unique fluorescence signal. Mismatches caused by SNPs that disrupt the binding of either of the probes can be used to detect mutant sequences present in a DNA sample.

There is a need for alternative methods for the detection of a plurality of nucleic acid hybrids in a single sample (multiplexing). There is a demand for such methods that are highly sensitive. For example methods to determine viral load of multiple viruses in a single sample that are able to reliably detect as few as 10 copies of a virus present in a body, tissue, fluid, or other biological sample would be in high demand. There is a great demand for methods to determine the presence or absence of nucleic acid sequences that differ slightly from sequences that might otherwise be present. There is a great demand for methods to determine the presence or absence of sequences unique to a particular species in a sample. There is also a great demand for methods that are more highly sensitive than the known methods, quantitative, highly reproducible and automatable.

It would be beneficial if another method were available for detecting the presence of a sought-after, predetermined target nucleotide sequence or allelic or polynucleotide variant. It would also be beneficial if such a method were

operable using a sample size of the microgram to picogram scale. It would further be beneficial if the various methods listed above were capable of providing multiple analyses in a single assay (multiplex assays). The disclosure that follows provides a wide variety of methods and materials for nucleic acid hybrid detection.

#### Summary of the Invention

The present invention contemplates methods and materials for determining the presence or absence of a nucleic acid hybrid. The convenient assays can use inexpensive reagents, such as unlabeled probes. The only enzyme necessary for most embodiments is a template-dependent polymerase. The assays are amenable to a very high degree of multiplexing and automation.

A sample that may contain a nucleic acid hybrid is maintained under conditions that permit pyrophosphorolysis. Analysis to determine whether or not pyrophosphorolysis occurs involves the addition of a nucleotide to a pyrophosphorylyzed 3'-terminal region in the nucleic acid hybrid. The pyrophosphorolysis followed by the incorporation of a nucleotide is the foundation of the present invention.

A contemplated reaction mixture for determining the presence or absence of a nucleic acid hybrid in a sample contains a sample that may have a nucleic acid hybrid, pyrophosphate, an enzyme that catalyzes pyrophosphorolysis of a 3'-terminal region of the nucleic acid hybrid, and a suitable nucleotide that can be incorporated into the 3'-terminal region in place of a pyrophosphorylyzed residue. The reaction mixture can include other components that do

not materially affect the pyrophosphorolysis and nucleotide incorporation in the reaction mixture.

A contemplated method comprises the steps that follow. A reaction mixture as described above is provided and maintained for a time period and under conditions that permit pyrophosphorolysis of the 3'-terminus of a strand of the nucleic acid hybrid to form a modified 3'-terminal region and the incorporation of a suitable nucleotide onto the modified 3'-terminus. The reaction solution after maintaining the reactions is referred to as a "treated sample". The treated nucleic acid sample is provided that may contain a nucleic acid hybrid. Many preferred embodiments involve the purposeful generation of specific nucleic acid hybrid formation using nucleic acid probes that hybridize to predetermined nucleic acid targets.

The sample is maintained under conditions that permit pyrophosphorolysis in the presence of pyrophosphate and in the presence of an enzyme capable of catalyzing the release of a nucleotide from the 3'-terminus of one of the strands of the nucleic acid hybrid when pyrophosphate is present. The treated sample is assayed to determine whether pyrophosphorolysis of the nucleic acid hybrid occurs. If pyrophosphorolysis occurs, then an appropriate nucleic acid hybrid is present. Conversely, if there is no pyrophosphorolysis, then the nucleic acid hybrid is not present in the sample. In preferred embodiments, a nucleotide is incorporated into the pyrophosphorylyzed nucleic acid strand in a template-dependent manner.

In preferred embodiments, the same enzyme catalyzes both specific reactions. In preferred embodiments, the enzyme performing both reactions is

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a template-dependant polymerase. In some preferred embodiments, the enzyme is a thermostable template-dependent polymerase.

Determination of whether or not a nucleotide is incorporated into the pyrophosphorylyzed 3'-terminus is done using any method of analyzing nucleic acids. One contemplated class of analytical methods involves the incorporation of labels to determine whether nucleotide incorporation occurs. Many such methods involve separation of free nucleotides from nucleic acid polymers, including but not limited to gel separation methods. Another contemplated class of analytical methods do not rely upon the incorporation of labels, but involve a determination of relative sizes of a nucleic acid probe. These methods also include but are not limited to gel separation methods. Another contemplated class of analytical methods may involve incorporation of a capture tag such that only products of the invention are selected from the group of probes. Alternatively, the probe or the template itself may contain a capture tag and any known method of analyzing DNA is useful to assess the pyrophosphorolysis/nucleotide incorporation step.

The present invention provides methods and materials useful for detecting nucleic acid hybrids in a variety of applications.

#### Detailed Description of the Invention

The present disclosure provides methods and materials for the qualitative or quantitative determination of a nucleic acid hybrid. A contemplated detection method builds upon the pyrophosphorolysis methods of nucleic acid hybrid detection disclosed in the earlier patents that are

cross-referenced above and incorporated in full herein by reference. Briefly, the invention contemplates the determination of whether a nucleic acid hybrid had been pyrophosphorylyzed to release nucleotides by the addition of a nucleotide to the pyrophosphorylyzed nucleic acid strand. Thus, a method embodiment of the invention comprises a depolymerization and incorporation of nucleotide (reincorporation).

The description that follows discusses in greater detail (A) the depolymerization and nucleotide incorporation reactions, (B) the substrates, (C) the enzyme, (D) the reaction conditions, (E) multiplex variations, (F) analytical output, (G) applications of the technology, and (H) kits.

A. Nucleic Acid Hybrid Detection Via Depolymerization and Incorporation of Nucleotide

The parent cases disclose materials and methods for nucleic acid detection via depolymerization of a nucleic acid hybrid. One of the depolymerization reactions disclosed therein is pyrophosphorolysis. Several methods were disclosed therein for ascertaining whether or not depolymerization of the nucleic acid hybrid had occurred, and thereby for detection of the nucleic acid hybrid. The present invention is concerned with ascertaining whether or not depolymerization of a nucleic acid hybrid occurred via incorporation of nucleotide, preferably catalyzed by the same enzyme.

i. Depolymerization

The present invention involves methods and materials for the pyrophosphorolysis of the 3' end of

a strand of a nucleic acid hybrid. An illustrative nucleic acid hybrid is shown below with a 5' overhang (though it also works at the 3' terminus of blunt-ended hybrids) where pyrophosphorolysis occurs in the presence of inorganic pyrophosphate and an enzyme that catalyzes pyrophosphorolysis, such as a template-dependent polymerase.



The pyrophosphorolysis reaction is separate and distinct from a "proofreading" reaction where an incorrectly incorporated nucleic acid base is excised from a growing nucleic acid chain. That "proofreading" function is associated with a separate and distinct 3' to 5' exonuclease activity. The proofreading function is so distinct from the polymerase reaction that in the Klenow fragment of *E. coli* DNA polymerase, it is possible to remove one enzyme activity without eliminating the other (e.g. "Klenow exo-minus"). Klenow exo-minus is capable of catalyzing both polymerization and pyrophosphorolysis.

The pyrophosphorolysis reaction is also different from an endonucleolytic cleavage, where a nucleic acid chain is cleaved, not at the end, but at an internal position. Typical endonucleolytic cleavages cleave both strands at a specific endonuclease recognition site.

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## ii. Incorporation of Nucleotide

The cross-referenced parent cases discuss various methods of detecting whether or not depolymerization (in this embodiment, via pyrophosphorolysis) of the nucleic acid hybrid occurs. The present invention focuses on embodiments wherein depolymerization is detected by the incorporation of one or more nucleotides in the depolymerized nucleic acid hybrid at the pyrophosphorylyzed end of the nucleic acid segment, as illustrated below.



In a typical polymerization reaction, a nucleotide triphosphate is added onto the 3'-terminus of a growing nucleic acid chain. In the case of DNA, a phosphonucleotide of a deoxyribonucleoside triphosphate (dNTP) is added with the production of inorganic pyrophosphate as a side product. In the case of RNA, a phosphonucleotide of a ribonucleoside triphosphate (NTP) is added with the production of inorganic pyrophosphate as a side product.

In contrast to polymerization, in a typical pyrophosphorolysis reaction, inorganic pyrophosphate is transferred to a phosphonucleotide as the phosphonucleotide is cleaved from the 3'-terminus of a nucleic acid chain. Pyrophosphorylyzed DNA releases a dNTP, and pyrophosphorylyzed RNA releases an NTP.





acid target sequence. As disclosed as an embodiment in the parent cases, depolymerization via pyrophosphorylation occurs most efficiently when the 3'-terminus of the probe is bound with a high degree of complementarity to the nucleic acid target sequence.

In a subset of embodiments for determining the presence or absence of a nucleic acid hybrid, a nucleic acid hybrid is provided. In some embodiments, a nucleic acid hybrid is separately previously formed, for example prior to admixture of an enzyme and pyrophosphate to form the reaction mixture. The formation of a nucleic acid hybrid (for example by providing an oligonucleotide probe capable of hybridizing with a nucleic acid target) is an additional step that is included in other embodiments. In other embodiments, a nucleic acid hybrid is formed *in situ* in the reaction mixture where depolymerization and incorporation of nucleotide occurs. However, hybrid formation is contemplated, for example by providing an oligonucleotide probe capable of hybridizing with a complementary nucleic acid target.

#### i. Nucleic Acid Probe

Some of the nucleic acid hybrid detection methods of the invention contemplate the formation of a specific nucleic acid hybrid using a nucleic acid probe that hybridizes to an essentially complementary nucleic acid target. A nucleic acid probe comprises single-stranded nucleic acid (e.g., DNA or RNA). A probe can be of varying lengths from a few to a few hundred nucleic acid residues, preferably from about 10 to 100 bases, more preferably from about 10 to about 65 bases, most preferably about 10 to 30 bases.

Preferably, a nucleic acid probe is designed to not hybridize with itself to form a hairpin structure in such a way as to interfere with hybridization of the 3'-terminal region of the probe to the target nucleic acid. Parameters guiding probe and primer design that are well known in the art apply equally well to the design or selection of probes for use with the present invention.

In a subset of embodiments of the invention, the nucleic acid probe is designed so that there can be no incorporation of a nucleotide into a strand of the nucleic acid hybrid until after pyrophosphorolysis. For example, where the 3'-terminal residue of a strand of the nucleic acid hybrid is a dideoxy (extension-blocking or chain-terminating) nucleic acid residue, extension is blocked. After such an extension-blocking residue is removed from the 3'-terminus via pyrophosphorolysis, sequence-specific nucleotide incorporation into the 3'-terminus of the pyrophosphorylyzed strand of the nucleic acid hybrid is carried out.

The invention contemplates the use of nucleotide analogs (including but not limited to inositol or any of the other available nucleotide analogs, several of which are listed in the World Intellectual Property Standard ST 25 (1998)) or modified nucleotides (including but not limited to biotinylated nucleotide as a capture label) in the construction of a nucleic acid probe.

In the design of a multiplex assay, variation of probe length, preferably by about 5 nucleic acid residues, can provide ready distinction between probes. Other methods to design readily distinguishable probes for a multiplex assay are also contemplated, including the use of a label elsewhere

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For example, a sample is contemplated that contains DNA at a site A and a site B. Further, it is contemplated that within a given population, variation in the DNA sequence is known to exist in the DNA at sites A and B such that SNPs exist at both of these sites resulting in an allele 1 and allele 2 at both of these sites.

Probes are designed such that a 15 bp probe hybridizes to allele 1 at site A in a manner that allows pyrophosphorylation followed by incorporation to take place; a 20 bp probe hybridizes to allele 2 at site A in a manner that allows pyrophosphorylation followed by incorporation to take place; a 25 bp probe hybridizes to allele 1 at site B in a manner that allows pyrophosphorylation followed by incorporation to take place; and a 30 bp probe hybridizes to allele 2 at site B in a manner that allows pyrophosphorylation followed by incorporation to take place. Reaction of these probes as contemplated in a subset of the conditions contemplated for the invention would produce: (a) labeled products of about 15 and 25 bp if the sample only contained allele 1 DNA at site A and site B; (b) labeled products of about 20 and 30 bp in length if the sample only contained allele 2 DNA at site A and B; or (c) labeled products of about 15, 20, 25 and 30 bp in length if the sample contained both allele 1 and allele 2 DNA at sites A and B, respectively. Expansion of this reaction design to



Intellectual Property Standard ST 25 (1998)) or modified nucleotides (including but not limited to biotinylated nucleotide as a capture label) in the construction of a nucleic acid target.

iii. Interrogation Position

Depolymerization reactions can be used to interrogate the identity of a specific base in a nucleic acid. For example, the identity of single base mutations, deletions, or insertions in a nucleic acid can be determined. Not all of the embodiments of the present invention utilize the interrogation position.

In one embodiment, each nucleic acid probe synthesized is substantially complementary to a target nucleic acid containing or suspected of containing a point mutation. It will be recognized that various hybridization conditions can be used, so as to vary the stringency at which hybridization occurs.

Depending on the length of the probe, the nucleotide content, and the stringency of the hybridization conditions, the probe can have mismatches in regions other than the 3'-terminal region and still hybridize sufficiently for the present invention, which is concerned with mismatches in the 3'-terminal region. In one preferred embodiment, the probe has only one base mismatch (at the single base of interest when it is not the probe designed to match that target exactly) with the target nucleic acid, and the mismatch is in the 3'-terminal region of the nucleic acid probe and the remaining bases of the probe are completely complementary to the nucleic acid target.

In preferred embodiments, a probe is designed to have a predetermined nucleotide at an interrogation position. When a complementary probe base pairs or hybridizes to a target nucleic acid, the base at an interrogation position aligns with the base in the nucleic acid target whose identity is to be determined under conditions such that base pairing can occur.

In preferred embodiments where the interrogation position is not at the 3'-terminal position, a probe is complementary to its predetermined target at all bases of the 3'-terminal region between an interrogation position and 3' end of the nucleic acid probe.

Typically, in a contemplated interrogation assay, the nucleic acid probes are designed so that the base at an interrogation position is complementary to the nucleotide at the predetermined position of the nucleic acid target (such as an allele), but not to other variations of that predetermined nucleic acid sequence, due to the

mismatch. Likewise, a second probe can be synthesized that is complementary at an interrogation position to the nucleotide at the predetermined position of the other variations of the predetermined nucleic acid sequence (such as a different allele at that site).

An example of an interrogation embodiment was discussed in the "Nucleic Acid Probe" section above using the designations allele 1 and allele 2. The position where the SNP occurs corresponds to the interrogation position of the nucleic acid probe.

iv. Complementarity and Mismatches

As noted in the parent applications, processes of the invention can be used to determine whether there is a match or a mismatch in the 3'-terminal region of a single or a few bases between a nucleic acid probe and a nucleic acid target sequence.

The distinction between a matched and mismatched base becomes less notable as a single mismatch is at a position further upstream from the 3'-terminal position. There is very little discrimination between a match and mismatch when a single mismatch is eleven to twelve residues from the 3'-terminal nucleotide position, whereas great discrimination is observed when a single mismatch is very near the 3'-terminus.

As disclosed in the parent application, in particular embodiments of the invention, it is desirable to include a second, destabilizing mismatch in or near the 3'-terminal region (about the last ten 3'-terminal nucleotides) of the probe, because it can enhance the discrimination between a match and a mismatch closer to the 3'-terminus (for example at



the last or penultimate residue in the 3'-terminal regions, as discussed above).

v. 3' Terminus of Detection Substrate

The preferred substrate for the depolymerization and polymerization reactions is a nucleic acid hybrid having a 3'-terminus that has a 5'-overhang on the opposing strand (a recessed 3'-terminus), as illustrated above. The pyrophosphorolysis is also effective on blunt-ended nucleic acid hybrids having neither a 3' nor 5' overhang at the nucleic acid hybrid end of interest.

There are other ways to provide such a substrate besides the addition of a nucleic acid probe to a solution that may contain a nucleic acid target. There may already be a duplex nucleic acid in a sample that has the appropriate overhang. An alternative way to generate an appropriate substrate is by endonucleolytic cleavage with an endonuclease that leaves the appropriate duplex ends, as discussed in the incorporated parent cases.

C. Depolymerization and Nucleotide Incorporation  
Enzyme

In some embodiments, the present invention contemplates carrying out pyrophosphorolysis and incorporation of nucleotide in the same solution in a single reaction vessel. In such an embodiment, it is preferable to balance the conditions between those that favor pyrophosphorolysis and those that favor polymerization (nucleotide incorporation) of the nucleic acid strand. Several of the Examples of the invention below illustrate such conditions.

In many of embodiments of the invention contemplated, an enzyme capable of catalyzing

pyrophosphorolysis is provided. In several embodiments, pyrophosphorolysis is followed by polymerization. In a subset of these embodiments, the enzyme catalyzing pyrophosphorolysis and polymerization is the same, and it is a template-dependent nucleic acid polymerase.

i. Enzymes

Template-dependent nucleic acid polymerases capable of pyrophosphorolysis include, but are not limited to, DNA polymerase  $\alpha$ , DNA polymerase  $\beta$ , T4 DNA polymerase, *Taq* polymerase, *Tne* polymerase, *Tne* triple mutant polymerase (Readase™, Promega Corp. Madison, WI), *Tth* polymerase, *Tvu* polymerase, *Ath* polymerase, *Bst* polymerase, *E. coli* DNA polymerase I, Klenow fragment, Klenow exo minus (exo-), AMV reverse transcriptase, RNA polymerase and MMLV reverse transcriptase. Most preferably, Klenow exo minus (Klenow exo-) or *Tne* triple mutant polymerase is utilized for DNA pyrophosphorolysis reactions because of their efficient utilization of 5' overhanging DNA ends.

The *Tne* triple mutant DNA polymerase is described in detail in WO 96/41014, whose disclosures are incorporated by reference, and its 610 residue amino acid sequence is provided as SEQ ID NO:35 of that document. That enzyme is referred to in WO 96/41014 as *Tne* M284 (D323A,D389A).

The thermostable polymerases are particularly preferred in embodiments of the present invention that utilize thermal cycling.

ii. Conditions for Enzyme Activity

The optimal conditions for polymerization reaction of the enzymes are well known in the art.

The Examples below provide a variety of useful buffers, pH, temperature and salt conditions.

A suitable nucleotide for incorporation

As such, an A will go opposite a T, and a G opposite a C, as usual, but also the invention contemplates that nucleotide analogs may be incorporated. Several nucleotide analogs are

presently known, and many of the most common ones are listed in the World Intellectual Property Standard ST 25 (1998). Other nucleotide analogs are known or may later be developed that would also be useful with the present invention and are presently contemplated. A labeled or otherwise modified nucleic acid residue is contemplated for use with the present invention. For example, the use of fluorescent or radiolabels is contemplated, as discussed herein. In addition, the use of capture labels, which as used herein refer to residues modified for attachment to a solid substrate, such as biotinylated residues that are captured on an avidin-derivatized support. Other such pairs as biotin/avidin are known in the art and are also contemplated for use with the present invention.

D. Conditions for Nucleic Acid Hybrid Detection

In preferred embodiments of the present invention, the same enzyme catalyzes both pyrophosphorolysis and polymerization. The reaction conditions are such that these opposite reactions can occur. The Examples below provide some illustrative conditions that permit both reactions to occur. A particular "balance" between pyrophosphorolysis and polymerization need not be struck between these two opposing enzyme activities. For example, the invention is also effective where the balance of conditions is closer to the polymerization side, yet sufficient depolymerization occurs to get an answer to the assay. Selection of conditions depends on the goals of the assay and many other factors in addition to the issue of what provides maximal polymerization or depolymerization activity.

Hybridization conditions can be empirically ascertained for a control sample for various time periods, pH values, temperatures, nucleic acid probe/target combinations and the like. Exemplary maintenance times and conditions are provided in the specific examples hereinafter and typically reflect low stringency hybridization conditions. In practice, once a suitable set of hybridization conditions and maintenance time periods are known for a given set of probes, an assay using those conditions provides the correct result if the nucleic acid target sequence is present. Typical maintenance times are about 5 to about 180 minutes.

In many of the contemplated processes of the present invention, a hybridization step occurs in the same solution under the same conditions at the same time as a depolymerization and a post-depolymerization polymerization step (in different probe/target hybrid complexes in that solution).

In an embodiment where a template-dependent polymerase catalyzes a pyrophosphorolysis and a polymerization reaction on a probe-target nucleic acid hybrid, the preferred conditions are, in general, slightly different from those preferred in the parent case, which focused primarily on carrying out the depolymerization.

#### i. Probe and Target Concentrations

In some preferred embodiments, oligonucleotide probes are typically utilized at about 100 ng to about 1  $\mu$ g per 20  $\mu$ L of reaction solution. That amount provides about 200 to about 1000 mass units of probe to about 1 mass unit of target weight.

In a preferred embodiment of the present invention, nucleic acid polymerase and pyrophosphate ( $PP_i$ ) or an analogue thereof, are added to a hybridized sample containing from less than about 100  $\mu$ g of target nucleic acid, to less than about 10  $\mu$ g of nucleic acid. Typical target nucleic acids are present at about 1 to about 5 ng in the sample to be assayed, with a target nucleic acid length of about 30 to about 1000 bp being preferred.

ii. Enzyme Concentrations

A depolymerizing enzyme is preferably present in an amount sufficient to pyrophosphorylyze a nucleic acid hybrid to form a modified 3'-terminus. A polymerizing enzyme is preferably present in an amount sufficient to add a suitable nucleotide to a modified 3'-terminus. Preferably the depolymerizing and polymerizing enzymes are the same enzyme. That amount can vary with the enzyme used, the pyrophosphorolysis temperature, the buffer, and the like. Typical buffer and temperature conditions are discussed above..

Examples of useful enzyme concentrations are found in the examples below and the parent cases. Preferably, enzyme concentrations range from about 0.25 to about 5 units (U) of a contemplated enzyme in a 20  $\mu$ L volume of reaction mixture. More preferably, for a typical reaction carried out in a 20  $\mu$ L volume, about 0.25 to about 1 unit (U) of a non-thermostable enzyme such as Klenow exo- is used. More preferably, for a typical reaction carried out in a 20  $\mu$ L volume, about 1 to about 5 U of the thermostable enzymes are used.

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Thermal cycling can be used to encourage dissociation of the longer probes after permitting some time for reaction. The examples below demonstrate that thermal cycling can eliminate the length effect. Such a result is particularly useful in a multiplex reaction where probe length is varied to more easily distinguish between several simultaneous assays.

Another technique that can assist with turnover of nucleic acid hybrids, notably in the context of probe:target hybrids, is to add RecA to the reaction mixture. The E. coli RecA protein (Promega Corporation M1691) binds cooperatively and stoichiometrically to single-stranded DNA and is active in strand exchange as a nucleoprotein containing one RecA monomer (38 kDa) per 3 bases of ssDNA.

#### E. Multiplex Nucleic Acid Detection

A multiplex method of this invention is used to determine the presence or absence of a plurality of predetermined (known) nucleic acid target sequences in a nucleic acid sample.

In a multiplex method a sample to be assayed is admixed with a plurality of nucleic acid probes. The admixing of the first step is typically carried out under low stringency hybridizing conditions to form a hybridization composition. In such a hybridization composition, the 3'-terminal region of the nucleic acid probes hybridize to a nucleic acid target sequence that may be present in the sample.

The hybridization composition is maintained under hybridizing conditions for a time period sufficient to form a treated sample that may contain

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a plurality of predetermined nucleic acid target sequences hybridized with their respective nucleic acid probes.

When a method of the present invention is used to determine whether a particular target sequence is present or absent in a sample to be assayed, the target may be absent. When the target is absent, the resulting treated sample does not contain an appropriate template for polymerization if there was no prior depolymerization. As a result, 3' terminal residues of the probe are not released, then there is no polymerization of the depolymerized probe, and the analytical output is at or near background levels.

A contemplated method is a multiplex assay in which a plurality of probes is utilized to determine whether one or more of a plurality of predetermined nucleic acid target sequences is present or absent in a sample. As discussed in the parent cases, the probes can be designed so that a positive analytical output indicates that the target is present, or so that a positive analytical output indicates that the target is absent. For example, an unlabeled probe can be pyrophosphorylyzed to form a modified 3'-terminal region and then a suitable nucleotide having a label can be incorporated into the modified 3'-terminal region to form an incorporated modified 3'-terminal region. Analysis for the label that gives a positive analytical output means that the target was present. Alternatively, a label on a probe can be removed by pyrophosphorolysis when the target is present, resulting in a modified 3'-terminus that no longer is associated with a label. After polymerization, an assay for the label

will result in a positive analytical output when the target is absent.

F. Analytical Output for Nucleic Acid Detection

The analytical output is used to determine whether or not pyrophosphorolysis has occurred. In many embodiments herein, analytical output indicated whether polymerization has occurred will indicate whether or not pyrophosphorolysis has occurred. Exemplary detection systems include various gel visualization methods, including fluorescence. These detection systems and others are discussed in the incorporated parent applications.

The fact that nucleotides were released (a qualitative determination), or even the number of nucleotides released (a quantitative determination) can be deduced through examination of the probe after depolymerization. The determination of the size of an oligonucleotide is well known in the art. For example gel separation and chromatographic separations are well known. Gel imaging techniques that take advantage of fluorescence and absorbance spectroscopy as well as radiographic methods have been described and are contemplated. Mass spectrometry of oligonucleotides is also becoming more common and are contemplated for use with the present invention.

In an embodiment of the present invention, the depolymerized probe is polymerized to incorporate one or more additional bases. An assay to determine whether the depolymerized probe is polymerized gives rise to analytical output that is used to determine whether the nucleic acid target sequence was present.

In one contemplated embodiment of the invention, the enzyme whose activity is to

depolymerize hybridized nucleic acid to release nucleotides from the probe 3'-terminal end is a template-dependent polymerase, as described above. In such an embodiment, the reverse of a polymerase reaction is used to depolymerize a nucleic acid probe, and the identifier nucleotide is released when the 3'-terminal nucleotide of the nucleic acid probe hybridizes with total complementarity to its nucleic acid target sequence. A signal confirms the presence of a nucleic acid target sequence that has the sequence sufficiently complementary to the nucleic acid probe to be detected by the process of the invention.

The magnitude of a contemplated analytical output under defined conditions is dependent upon the amount of released nucleotides. Where an identifier nucleotide is released, re-polymerization of the remaining, depolymerized probe can be analyzed to provide an analytical output that has a value greater than background. Where an identifier nucleotide is not released--either because the target sequence was not present in the original sample or because the probe did not match sufficiently to become a pyrophosphorolysis substrate--the analytical output is substantially at a background level.

In some cases, it may be beneficial to enrich the nucleic acid target for hybrid formation within the nucleic acid sample. Many illustrative embodiments described in the parent applications involve detection of a gene or marker from within a massive genome, such as nucleic acid target sequences associated with blood coagulation (such as those probes associated with the Factor V Leiden mutation), probes specific to various classes of animals (such as bovine, murine, homo sapiens), congenital adrenal

hyperplasia (CAH), sequences associated with cystic fibrosis, cancer-associated sequences (e.g. *Bcr/Ab1*).

In such embodiments, it is beneficial to enrich the target sequence within the sample. A target nucleic acid sequence is amplified prior to use of the present invention. Several methods are known in the art to amplify a region of DNA. These include polymerase chain reaction (PCR), ligase chain reaction (LCR), repair chain reaction, amplification of transcripts, self-sustained sequence replication (3SR), ligation activated transcription (LAT), strand displacement amplification (SDA) and rolling circle replication. A claimed process contemplates prior treatment of a nucleic acid sample using any amplification method known in the art at the time of practicing a claimed process to detect the presence of a nucleic acid target in the sample.

#### i. Separation for Analysis

A great majority of the nucleic acid hybrid detection methods disclosed herein involve a separation step for analysis, most typically separation of probes incorporating a nucleotide from free nucleotides. This includes the embodiments with and without the incorporation of labels. Separation methods of the art are contemplated for use herewith, which may include, but are not limited to, electrophoretic separation or separation on a solid support (both of which are illustrated below).

Shrimp alkaline phosphatase or a related enzyme may be useful at times to alter the mobility of the nucleotides on a separation gel, as done in an example below to ascertain whether any bands were obscured. Shrimp Alkaline Phosphatase (SAP from *Pandalus borealis*; Promega Corporation M8201)

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catalyzes the dephosphorylation of 5' phosphates from DNA and RNA. Unlike Calf Intestinal Alkaline Phosphatase, SAP is completely and irreversibly inactivated by heating at 65 C for 15 minutes. Alkaline phosphatases are also useful to prevent recircularization and religation of linearized nucleic acid by removing phosphate groups from both 5'-termini and may also be used for the dephosphorylation of 5' phosphorylated ends of DNA or RNA for subsequent labeling with [32P]ATP and T4 Polynucleotide Kinase. SAP is active on 5' overhangs, 5' recessed and blunt ends. The pyrophosphorolysis and nucleotide incorporation (polymerization) reactions of the present invention act on the 3'-terminus of a nucleic acid; the alteration of the 5' terminus has no effect on the pyrophosphorolysis and polymerization.

Some embodiments do not require separation, such as a fluorescence energy transfer embodiment where a probe will not affect the fluorescence of a hybridizing co-analyte (or vice versa) until the fluorescence-affecting moiety (or fluorophore) has been incorporated into the probe according to the invention.

#### ii. Absorbance Spectroscopy

An absorbance spectrographic analysis step is contemplated to provide an analytical output, thereby provide for the determination of the presence or absence released identifier nucleotide or an incorporated suitable nucleotide, thus indicating the presence or absence of said nucleic acid target sequence. This embodiment contemplates the separation of a reaction mixture that has been treated with a depolymerizing amount of a

pyrophosphorylizing enzyme followed by polymerization and then visualization, such as on a gel. Such visualization can be by the human eye, without the need for extra equipment, such as a spectrograph.

iii. Fluorescence Spectroscopy

A wide variety of fluorescence detection methods can be used herein. In one exemplary contemplated method, an identifier nucleotide includes a fluorescent label. An identifier nucleotide can be fluorescently labeled prior to, or after, release of the identifier nucleotide. In an alternative embodiment when the nucleotide is fluorescently labeled, the analytical output is obtained by mass spectrometry.

In a preferred embodiment of the invention, the fluorescent label is part of a fluorescent analog of a nucleotide. Fluorescent nucleotide analogs are widely known and commercially available from several sources. An exemplary source is NEN Life Science Products (Boston, Massachusetts), who offer dideoxy-, deoxy-, and ribonucleotide analogs a labeled with fluorescein, coumarin, tetramethylrhodamine, naphthofluorescein, pyrene, Texas Red®, and Lissamine™. Other suppliers include Amersham Pharmacia Biotech (Uppsala, Sweden; Piscataway, New Jersey) and MBI Fermentas, Inc. (Amherst, New York).

The fluorescent label may be present on the released identifier nucleotide, or it may be present elsewhere on the interrogation probe as a marker of that probe or it may be in the incorporated suitable nucleotide. The presence of differently fluorescing analogs on different probes provides a ready means to increase the number of probes analyzable in one experiment (multiplexing). The interrogation probe

may be labeled with more than one fluorescent probe thus generating mixtures of multiply-labeled probes that further increases the number of interrogation probes that can be analyzed in a single experiment.

An advantage to using fluorescent labels and fluorescence spectroscopy analysis is that there are multiple different labels available. Such different labels would be particularly useful in a multiplex embodiment of the invention. Different fluorescent labels would be used in different probes, so that the detection of a particular fluorescently-labeled nucleotide analog as a released identifier nucleotide or as an incorporated suitable nucleotide or elsewhere in the nucleic acid hybrid or nucleic acid probe could be used to deduce which nucleic acid targets are present.

For example, fluorescein has a 488 nm maximum excitation and 520 nm maximum emission wavelength, whereas rhodamine (in the form of tetramethyl rhodamine) has 550 nm maximum excitation and 575 nm maximum emission wavelength. A fluorescence detector provides an excitation source and an emission detector. The emission wavelengths of 520 nm and 575 nm are easily distinguishable using fluorescence spectroscopy.

On a per molecule basis, fluorescence spectroscopy is about 10-fold more sensitive than absorbance spectroscopy. A very wide variety of fluorescence spectroscopy-based detectors are commercially available for reading fluorescence values of single tubes, flow cells and multi-well plates, among others. For example, Labsystems Multiskan models of microplate readers are widely available with a spectral range of 400 to 750 nm, and filters for 340, 405, 414, 450, 492, 540, 620, and

690 nm (e.g. Fisher Scientific, Pittsburgh, Pennsylvania).

It is contemplated that an incorporated suitable nucleotide could be labeled before or after incorporation using cross-linking chemistry well known in the art with commercially available reagents. For example, fluorescein isothiocyanate and rhodamine B isothiocyanate are both available from Aldrich Chemical Company (Milwaukee, Wisconsin). References to fluorescein isothiocyanate's use in labeling biological molecules include *Nature*, **193**:167 (1962), *Methods Enzymol.* **26**:28 (1972), *Anal. Biochem.*, **57**:227 (1974), *Proc. Natl. Acad. Sci., U.S.*, **72**:459 (1975).

It is contemplated that for many embodiments of the invention, it is useful to separate fluorescent nucleotides from those bound to an oligonucleotide, such as a probe. Thus, the separation techniques well known in the art and discussed above are useful with such an embodiment, including gels, capillary systems or an HPLC fitted with a fluorescence detector. The enhanced sensitivity of fluorescence relative to other spectroscopic techniques can be used to increase the sensitivity of a detection or quantification process of the invention.

#### G. Applications of the Detection Technology

Several applications of probe hybridization methods of determining the presence or absence of predetermined nucleic acid target sequences are disclosed in the parent cases. The presently disclosed method of ascertaining whether or not pyrophosphorolysis occurred are applicable to those

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methods, including the analysis of single nucleotide polymorphisms.

Methods and materials of the present invention are useful for a wide variety of applications. One notable embodiment is the detection of the presence or absence of a predetermined nucleic acid sequence in a sample using oligonucleotide probes capable of hybridizing with the predetermined nucleic acid sequence. The present invention contemplates embodiments for the detection of predetermined sequences, sensitive enough to detect a single amino acid variation in the predetermined sequence, such as a single nucleotide polymorphism (SNP). The present invention contemplates an embodiment having a multiplex format, where the presence or absence of several predetermined nucleic acid sequences may be determined at once in the same nucleic acid sample at the same time.

In yet another preferred embodiment, the probe-mediated specific nucleic acid detection method of the present invention can be used to simply identify or detect a nucleic acid of interest. For this method, nucleic acid probes (e.g., DNA or RNA) are utilized, each of which is substantially complementary to its respective target nucleic acid, which can be RNA or DNA. In a particularly preferred embodiment, each nucleic acid probe is entirely complementary to its target nucleic acid. A nucleic acid probe comprises single-stranded nucleic acid (e.g., DNA or RNA). The probe can be of varying lengths, preferably from about 10 to about 1000 bases, most preferably about 10 to 100 bases.

The ability to interrogate the identity of a specific base in a nucleic acid also permits

In preferred embodiments of this method, nucleic acids with substantially identical sequences from at least two species or alleles are detected. The region of identity (target nucleic acid sequence) contains at least a single nucleotide mismatch between the species or alleles in at least one predetermined position and also contains a 3' end and a 5' end or the identification of a nucleic acid sequence unique to each species to be identified.

Using a contemplated method, one can illustratively determine whether a human patient, for example, has the Leiden Factor V mutation, a mutant  $\beta$ -globin gene, the cystic fibrosis-related gene in the region of the delta 508 allele, a mutation in a prothrombin gene, congenital adrenal hyperplasia, a translocation that takes place in the region of the *bcr* gene along with involvement of a segment of the *abl* gene, the number of repeated sequences in a gene such as are present in THO 1 alleles or the TPOX alleles, as well as the loss of heterozygosity of the

Another embodiment of the invention uses the nucleic acid hybrid analysis to determine nucleic acid sequences, through sequencing using pyrophosphorylation-promoted end reduction followed by base addition. Pyrophosphorolysis of the nucleic acid hybrid cleaves nucleotides from the 3'-terminus

of one of the nucleic acid strands. Pyrophosphorolysis can continue after the first nucleotide is removed from the original hybrid, and then there is a new 3'-terminal residue. As earlier described, under appropriate equilibrium conditions between polymerization and pyrophosphorolysis, a suitable nucleotide can be incorporated into the depolymerized hybrid whenever that suitable nucleotide can form the appropriate base pair. In some sequencing embodiments, the nucleic acid hybrid, or a strand thereof is labeled. In some sequencing embodiments, the suitable nucleotide is labeled.

For example, when the suitable nucleotide is a fluorescent dideoxycytosine residue, then that labeled C can be incorporated into the hybrid at the 3'-terminus wherever a C is appropriate. The scheme below is a schematic representation of this process with SEQ ID NO:43 and its complements, however the pyrophosphate and released nucleotides and suitable nucleotides are omitted for brevity.

5'...GCATCGATCCAAGCAGACATGCA...-3'  
3'                  TTCGTCTGTACGT...-5'

Enzyme



5'...GCATCGATCCAAGCAGACATGCA...-3'  
3'                  GTCTGTACGT...-5'  
and  
5'...GCATCGATCCAAGCAGACATGCA...-3'  
3'                  TGTACGT...-5'

Enzyme



5'...GCATCGATCCAAGCAGACATGCA...-3'  
3'                  \*CGTCTGTACGT...-5'  
and  
5'...GCATCGATCCAAGCAGACATGCA...-3'  
3'                  \*CTGTACGT...-5'

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This embodiment of the invention does not require a probe to hybridize with the nucleic acid sequence of interest. For example, a cleavage of a target DNA to produce a duplex strand with a substrate 3'-terminus is also contemplated.

A multiplex embodiment is contemplated wherein multiple nucleotide ladders can be formed in the same reaction. For example by providing a differently labeled thymidine residue, the T ladder can be distinguished from the C ladder described above.



Thus, a sequencing ladder can be built up using the pyrophosphorolysis and polymerization methods of the invention.

In a subset of the sequencing embodiments of the invention, it is contemplated that the pyrophosphorylyzed nucleic acid strand be separable, for example using a capture label, such as biotin. The incorporated suitable nucleotide is derivatized with the capture label, or the capture label is elsewhere on the nucleic acid hybrid.

For example, in one embodiment of this concept, biotinylated probes are used to microsequence a nucleic acid target. This method embodiment of the invention is carried out and the 3'-terminus of the probe is pyrophosphorylyzed and one or more suitable nucleotides is incorporated into

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the probe. The product probes of the reaction are separated from the reaction mixture by contacting the reaction mixture with an avidin-derivatized support. The probes are then analyzed to deduce the sequence of the target nucleic acid.

For example, two alleles of interest are as follows.

Allele B: 5'-GGA CCC TTA CCA-

of a suitable nucleotide. An allele A homozygote would show strong signals at lengths of primer -0, primer -2, and possibly at the primer -6 and primer -7. A homozygote of Allele B would show another band of equal strength at primer -1. A heterozygote would have the band at primer -1 that the Allele B homozygote would have but it would be half the strength of the primer -0 and primer -2 bands. Thus, in a single reaction mixture the genotype of the sample at the SNP site can be determined and the fact that the interrogation was taking place at the desired locus is confirmed by the other diagnostic bands formed.

#### H. Kits for Nucleic Acid Detection

Other embodiments of the invention contemplate a kit for determining the presence or absence of a nucleic acid hybrid, or a predetermined nucleic acid target sequence(s) in a nucleic acid sample. Such a kit comprises an enzyme that has pyrophosphorolysis activity and at least one nucleic acid probe, said nucleic acid probe being complementary to a nucleic acid target sequence.

Preferably the enzyme whose activity is to release nucleotides in the kit is a template-dependent polymerase that, in the presence of pyrophosphate ions, depolymerizes hybridized nucleic acids whose bases in the 3'-terminal region are complementary.

In a preferred embodiment, the enzyme capable of catalyzing pyrophosphorolysis is, but is not limited to *Taq* polymerase, *Tne* polymerase, *Tne* triple mutant polymerase, *Tth* polymerase, *Tvu* polymerase, *Ath* polymerase, *T4* DNA polymerase, *Klenow* fragment, *Klenow* exo minus, *E. coli* DNA polymerase I,

The depolymerizing enzyme is preferably provided in a kit in a form that is convenient to introduce into a contemplated assay in the appropriate concentration and in a form that provides sufficient unit activity. An enzyme of a kit is typically present in an amount that conveniently permits the distribution of about 0.1 to 100 U per reaction; in particularly preferred embodiments, the concentration is about 0.5 U/reaction. In a preferred kit, an amount of enzyme sufficient to carry out at least one assay, with its controls is provided.

It is to be understood that such a kit is useful for any of the methods of the present invention. The choice of particular components is dependent upon the particular method the kit is designed to carry out (several are described above). Additional components can be provided for detection of the analytical output, such as labeled nucleotides for incorporation by polymerization into the depolymerized probe.

In one embodiment, a kit has a plurality of different nucleic acid probes that comprise fluorescent labels. In a method using such a kit, either the 3'-terminal region having incorporated suitable nucleotide or released identifier nucleotide or elsewhere on the remaining probe is detected using fluorescence spectroscopy, depending on the location of the fluorescent label within the probe. In an alternative embodiment, the released nucleotides are separated from depolymerized probes, and the remaining probe or released nucleotide is fluorescently labeled. Such an embodiment



contemplates the provision of fluorescent labels and/or a magnetic nucleic acid separation medium. The above fluorescent embodiments contemplate that the fluorescent labels are distinguishable.

In another embodiment, a kit has nucleic acid probes that comprise a non-natural nucleotide analog as an identifier nucleotide. In a contemplated method using such a kit, the depolymerization is assayed for using mass spectrometry.

The kit optionally further comprises instructions for detecting said nucleic acid by depolymerization. The instructions present in such a kit instruct the user on how to use the components of the kit to perform the various methods of the present invention. These instructions can include a description of the detection methods of the invention, including detection by luminescence spectroscopy, mass spectrometry, fluorescence spectroscopy, and absorbance spectroscopy.

In one embodiment, the invention contemplates a kit for determining the presence or absence of a plurality of predetermined nucleic acid target sequences in a nucleic acid sample comprising the following components: an enzyme whose activity in the presence of pyrophosphate is to release identifier nucleotide as a nucleoside triphosphate from hybridized nucleic acid probe; pyrophosphate; and a plurality of nucleic acid probes, wherein the nucleic acid probes are complementary to their respective predetermined nucleic acid target sequence.

The enzyme whose activity in the presence of pyrophosphate is to release identifier nucleotide is as described hereinabove. Preferably, the

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The examples of the invention that follow are intended to illustrate selected embodiments of the present invention and should not be construed as limiting the present invention to these embodiments.

The examples of the invention that follow are intended to illustrate selected embodiments of the present invention and should not be construed as limiting the present invention to these embodiments.

Example 1: Fluorescent Nucleotide Incorporated into a Probe Modified by Pyrophosphorylation

In the present example, a probe annealed to a target was pyrophosphorylated and then elongated by a single dideoxynucleotide. The products were examined and demonstrated to have incorporated the expected nucleotide.

Probe oligonucleotide P1A (SEQ ID NO:1) and probe oligonucleotide P2a (SEQ ID NO:2) were designed to contain a 3' cytosine residue and to hybridize with exact homology to targets T1 and T2, respectively. These probe oligonucleotides function as probes in a pyrophosphorylation reaction for which oligonucleotide T1 (SEQ ID NO:3) or T2 (SEQ ID NO:4) is the target.

P2a	5' - CGGTGCGGGCCTCTAC	SEQ ID NO:2
P1a	5' - CGGTGCGGGCCTCTTC	SEQ ID NO:1
T1	5' - ATAGCGAAGAGGCCCGCACCGATCGCCCTTC CCAACAGTTGCGCAGCCTGAATGGCGAATGGAAA	SEQ ID NO:3
T2	5' - ATAGCGTAGAGGCCCGCACCGATCGCCCTTC CCAACAGTTGCGCAGCCTGAATGGCGAATGGAAA	SEQ ID NO:4

Two microliters of 1 mg/ml target T1 and T2 were each diluted into 500  $\mu$ l of denaturation

solution (0.06 N NaOH) and 498 microliters of NANOPURE (Millipore Corp.) water yielding a final concentration of 2  $\mu\text{g}/\text{ml}$ .

Probe oligo P2a and P1a stock solutions of approximately 200  $\mu\text{M}$  were diluted to make working probe solutions. Working probe solutions were assembled by adding 5  $\mu\text{l}$  of oligonucleotide P2a or P1a stock solution to 45 microliter of water and 50  $\mu\text{l}$  of MOPS-A (200 mM MOPS, 20mM  $\text{MgCl}_2$ , pH 6.1) yielding a final concentration of 0.05  $\mu\text{g}/\mu\text{l}$ .

Probe control solution ( $P_{\text{control}}$ ) was assembled by mixing 50  $\mu\text{l}$  of MOPS-A with 50  $\mu\text{l}$  of water. Target control solution ( $T_{\text{control}}$ ) was assembled by mixing 50  $\mu\text{l}$  of denaturation solution with 50  $\mu\text{l}$  of water.

Tubes were prepared in triplicate to contain 10  $\mu\text{l}$  of either target T1 or target T2 or  $T_{\text{control}}$ . To each tube was added 10  $\mu\text{l}$  of probe P1a or P2a or  $P_{\text{control}}$  such that there were ultimately tubes containing each target or control with each probe or control.

The following Master Mix was assembled:

190 $\mu\text{l}$	water
4 $\mu\text{l}$	READASE™ Polymerase (Promega Corp. EAP2)
12 $\mu\text{l}$	$\text{NaPP}_i$ (Promega Corp. C113)
5 $\mu\text{l}$	200mM MOPS-A, pH 6.6
12 $\mu\text{l}$	Fluorescein-12-ddCTP (NEL 400, Lot 090107)

Ten microliters of the Master Mix was added to each of the tubes and they were incubated at 55°C, for one hour.

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Two microliters of reaction products were combined with 1  $\mu$ l of 6X gel loading buffer and loaded onto a pre-cast 20% acrylamide TBE gels (Invitrogen, Novex™, EC6315 or EC63155). The running buffer is 1X TBE diluted from the 10X stock (Promega, AA637). The gel was run until the orange G front was ~3/4 down the gel. The gels were then scanned using a Fluorimager™ and analyzed using Imagequant™ analysis software (both from Molecular Dynamics).

The results show that the negative controls, where there was no target present in the reactions, generated no specific bands on the gel. In addition, no products were seen where there was a mismatched base between the target and primer. When the matching probe and target were present (target T1 and probe P1a or target T2 and probe P2a), there were specific bands indicating incorporation of fluorescently labeled cytosine. Two labeled products are present, indicating pyrophosphorolytic activity past the first three nucleotides to the second cytosine in the probe. A high molecular weight band was also seen and was believed to be due to the non-denatured target:probe pair.

This example demonstrates that fluorescent oligonucleotides can be incorporated with high fidelity into a probe that was modified by pyrophosphorolysis.

Example 2: Fluorescent Nucleotide Incorporated  
into a Probe When Target is Present

In the present example, a specific target is detected when a fluorescent nucleotide is incorporated into a probe, which can happen only when the target complementary to the probe is present and

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the probe is pyrophosphorylyzed prior to incorporation of a single, labeled dideoxynucleotide.

Probe oligonucleotide P1a (SEQ ID NO:1), probe oligonucleotide P1b (SEQ ID NO:5), and probe oligonucleotide P1c (SEQ ID NO:9) were dissolved to create a solution of approximately 200µM in oligonucleotide.. The 3'-terminus of P1b and P1c hybridize with exact homology to a segment of target oligo T1. P1c hybridizes with both T1 and T2 to the portion of those targets where they are identical, and creates a hybrid structure having a base pair mismatch 15 residues from the 3'-terminus of the P1c. Probe oligos P1a and P1b each have terminal cytosines; P1c does not. Probe oligonucleotide P1b\* (SEQ ID NO:7) can hybridize with exact homology to a segment of target oligo T1 except that it contains a one base mismatch.

Probe oligonucleotide P2a (SEQ ID NO:2), probe oligonucleotide P2b (SEQ ID NO:6) each can hybridize with exact homology to a segment of target oligo T2 and both probe oligos have a terminal cytosine. Probe oligonucleotide P2b\* (SEQ ID NO:8) can hybridize with exact homology to a segment of target oligo T2 except that it contains a one base mismatch and it also has a 3' terminal cytosine.

Probe P1b	HO-CGATCGGTGCGGGCCTCTTC	SEQ ID NO:5
Probe P2b	HO-ATCGGTGCGGGCCTCTAC	SEQ ID NO:6
Probe P1b*	HO-CGATCGGTGCGGGCCTCTTC	SEQ ID NO:7
Probe P2b*	HO-ATCGGT <u>AC</u> GCGGCCTCTAC	SEQ ID NO:8
Probe P1c	HO-TTCGCCATTGAGGCAGCGCAACTG	SEQ ID NO:9

The target oligos were diluted by mixing 10 µl of target oligo stock solution, 450 µl NANOPURE™ (Millipore Corp.) water, and 500 µl of denaturation

The probe oligonucleotides were diluted with water to obtain a 200  $\mu\text{M}$  probe oligo stock solution. Working probe solutions were made by mixing 5  $\mu\text{l}$  of probe oligo stock solution, 45  $\mu\text{l}$  water, and 50  $\mu\text{l}$  of MOPS-A (200 mM MOPS, 20mM  $\text{MgCl}_2$ , pH 6.1), to a final concentration of 10  $\mu\text{M}$ .

Fresh Master Mix was prepared as described in Example 1. The Master Mix (10  $\mu$ l) was added to each of the 14 tubes and the tubes placed at 55°C for 70 minutes. Tubes were removed from heat and loaded onto a gel, as follows: two microliters of reaction products were combined with 1 microliter of 6X gel loading buffer and loaded onto a pre-cast 20% acrylamide TBE gels (Invitrogen, NOVEX™, catalog# EC6315 or EC63155). The running buffer was 1X TBE diluted from the 10X LSS stock (Promega, AA637). The gel was run until the orange G front is ~3/4 down the gel. The gels were then scanned using a FLUORIMAGER™ and analyzed using IMAGEQUANT™ analysis software (both from Molecular Dynamics).

The results showed that negative controls, where the combination was a target with a probe that was not complementary, did not generate a labeled reaction product. Where probes having a 3'-terminal cytosine that can hybridize to a target with total

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P3a	HO-ACGCAAACCTTTATCA	SEQ ID NO:10
P3b	HO-TAAGGACGCAAACCTTTATCA	SEQ ID NO:11
P3c	HO-ACAGCTAAGGACGCAAACCTTTATCA	SEQ ID NO:12
P3d	HO-TAGAAATACCACAGCTAAGGACGCAA ACTTTATCA	SEQ ID NO:13
P3e	HO-AACTTACCAGTAGAAATACCACAGCT AAGGACGCAAACCTTTATCA	SEQ ID NO:14
P3f	HO-GTAGTAATTGAACTTACCAGTAGAAA TACCACAGCTAAGGACGCAAACCTTTATCA	SEQ ID NO:15
P3g	HO-ACACCAAAATGTAGTAATTGAACTTA CCAGTAGAAATACCACAGCTAAGGACGCA AACTTTATCA	SEQ ID NO:16
T3	HO-AGGCTCCCCTTGATAAAGTTTGCGTC CTTAGCTGTGGTATTTCTACTGGTAAGTT CAATTACTACATTTTGGTGTGGATG	SEQ ID NO:17

The target T3 was diluted with Nanopure™ (Millipore Corp.) water to obtain a target oligo stock solution of 1 µg/ml. Working solution was made by mixing 5 µl of target oligo stock solution, 95 µl Nanopure™ (Millipore Corp.) water, and 100 µl of denaturation solution [see Example 1] to a 0.025 µg/ml final concentration. A target control (T<sub>control</sub>) solution was made that lacked any oligo.

Each probe nucleotide (1 mg/ml) was diluted into 50 µl of 200 mM MOPS, 20 mM MgCl<sub>2</sub>, pH 6.2 and water to produce 100 µl of working probe solution. Due to the various sizes of these probes, 5 µl of 1 mg/ml oligonucleotide stock was used for P3a, P3b and P3c; 10 µl of 1 mg/ml oligonucleotide stock for P3d, P3e and P3f; and 15 µl of 1 mg/ml oligonucleotide stock was used for P3g to create the working probe solutions.

Sixteen tubes were prepared to contain 10  $\mu$ l of target. Eight tubes were further prepared to contain 10  $\mu$ l of one probe (P3a, P3b, P3c, P3d, P3e, P3f or P3g) such that there was ultimately one tube containing target and each probe, plus a control tube containing only target and probe control solution (50  $\mu$ l water and 50  $\mu$ l MOPSA). There were 4 additional tubes containing 10  $\mu$ l of probe P3b and 4 additional tubes containing 10  $\mu$ l of probe P3g .

Master Mix for control reactions without a template-dependent polymerase was prepared as described in Example 1 and labeled MM-C. A second Master Mix for reactions to contain a template-dependent polymerase, (MM-D) was prepared by combining 210  $\mu$ l of Nanopure™ (Millipore Corp.) water with 4  $\mu$ l of Readase™ polymerase and 5  $\mu$ l of MOPS-A. In addition, a 100 mM solution of ATP (E601B, 11228511) was diluted 100 fold into Nanopure™ (Millipore Corp.) water. The dilute ATP solution was used to form ATP/RecA solution, containing 5  $\mu$ l of 1mM ATP, 5 $\mu$ l thermostable RecA protein and 90  $\mu$ l of Nanopure™ (Millipore Corp.) water.

The reaction tubes were then further prepared as follows: 10  $\mu$ l of MM-C was added to tubes 1 through 8; tubes 9 and 13 received 7 $\mu$ l of MM-C and 3  $\mu$ l of MM-D; tubes 10 and 14 received 3  $\mu$ l of MM-C and 7  $\mu$ l of MM-D; tubes 11 and 15 received 1 $\mu$ l of MM-C and 9  $\mu$ l of MM-D such that tubes 9-11 and 13-16 had decreasing amounts of labeled nucleotide. Tubes 12 and 16 duplicated tubes 2 and 7 respectively except that they also received 10  $\mu$ l of MM-C and 1  $\mu$ l of ATP/RecA solution. All tubes were incubated at 55°C, and then placed at -20°C.

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To visualize results, two microliter of reaction products were combined with 1 microliter of 6X gel loading buffer and loaded onto a pre-cast 20% acrylamide TBE gels (Invitrogen, Novex™, EC6315 or EC63155). The running buffer was 1X TBE diluted from the 10X LSS stock (Promega, AA637). The gel was run until the orange G front is about three-fourths of the way down the gel. They were then scanned using a Fluorimager™ and analyzed using the Imagequant™ analysis software (both from Molecular Dynamics).

The results show reaction products decreasing in mobility as the primer increases in size. The results further show that as the concentration of pyrophosphate and labeled dNTP decreases, so does the strength of the product signal. These results also show that for very long probes or for a very low concentration of label, RecA protein can enhance the level of product.

Example 4: Specific Label Incorporation in the  
Presence of Other Probes

In the present example, up to four different probes were pyrophosphorylyzed and then a single dideoxynucleotide was incorporated into each pyrophosphorylyzed probe. The products were examined and demonstrated to have incorporated the expected nucleotide regardless of presence of non-homologous probes present in the reaction mixture. The present example further shows that specific products can be prepared with high fidelity in the presence of multiple probes and multiple targets.

Probe oligonucleotide P1a (SEQ ID NO:1) can hybridize with exact homology to a segment of target oligo T1 (SEQ ID NO:3). P2b\* (SEQ ID NO:8) can hybridize with homology to a segment of target oligo

T2 (SEQ ID NO:4), except there is a single mismatch outside of the 3'-terminal region. Probe P3d (SEQ ID NO:13) can hybridize with total complementarity to a segment of target T3 (SEQ ID NO:17). The probes vary in total length from 16 to 35 base pairs.

Working probe solution was prepared as earlier described, mixing 5  $\mu$ l of probe oligo stock solution (1 mg/ml), 45 $\mu$ l Nanopure™ (Millipore Corp.) water, 50  $\mu$ l of MOPS-A (200 mM MOPS, 20mM MgCl<sub>2</sub>, pH 6.2). Mixed probe oligo solution, (P1a+P2b\*+P3d), was comprised of 5 $\mu$ l of each probe solution, 35 $\mu$ l of water and 50  $\mu$ l MOPS-A.

Working target oligo solution was prepared as earlier described, mixing 5  $\mu$ l of target oligo stock solution, 95  $\mu$ l Nanopure™ (Millipore Corp.) water, and 100  $\mu$ l of denaturation solution (Promega Corp., Lot 12754503) to a final concentration of 2  $\mu$ g/ml. Mixed target oligo solutions, (T3+T1) and (T3+T2) were each comprised of 5 $\mu$ l of each of targets T3 and T1 or T3 and T2, respectively, with correspondingly less water to a total volume of 200 $\mu$ l. Mixed target oligo solution (T1+T2+T3) was comprised of 5 $\mu$ l of target T3, 2.5 $\mu$ l each of T1 and T2, with corresponding less water to a total volume of 200 $\mu$ l.

Three tubes were prepared to contain 10  $\mu$ l of a probe solution and 10  $\mu$ l of its matching target. Two tubes were prepared to contain 10  $\mu$ l solution (T3+T1) and 10  $\mu$ l of probe P1a and 10  $\mu$ l (P1a+P2b\*+P3d), separately. Two tubes were prepared to contain 10  $\mu$ l solution (T3+T2) and 10  $\mu$ l of probe P2b\* and 10  $\mu$ l (P1a+P2b\*+P3d), separately. Eight

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tubes were prepared to contain 10  $\mu$ l solution (T1+T2+T3). Three (T1+T2+T3) tubes were prepared to contain 10 $\mu$ l of the individual probe solutions (P1a, P2b\* or P3d). Four (T1+T2+T3) tubes were prepared to also contain 10  $\mu$ l of (P1a+P2b\*+P3d). There was ultimately one tube containing each probe and target combination, plus tubes containing only probe with mixed targets, and multiple probes and targets together.

Master Mix was prepared as previously described in Example 1. Ten microliter of the Master Mix was added to each of the fifteen tubes. Readase™ polymerase was serially diluted four times in 1% MOPS-A to yield 1:2, 1:4, 1:8 and 1:16 dilutions. One microliter of each of these serial dilutions was added to 4 of the tubes containing (T1+T2+T3)/(P1a+P2b\*+P3d). All 15 tubes are placed at 55°C for one hour.

To visualize results, two microliters of reaction products were combined with 1 microliter of 6X gel loading buffer and loaded onto a pre-cast 20%acrylamide TBE gels, as previously described. Gels were then scanned and analyzed as previously described.

The results show reaction products for matching probe:target pairs. In reactions containing both matching and non-matching probe:target combinations, products were seen when a probe was present with both a matching and a non-matching target; only products from combinations without a mismatched base near the 3'-end of the probe were generated. Reaction products were also seen when numerous specific and non-specific reaction reagents were present, and only specific products were generated. The mismatch present on probe oligo P2b\*

had no effect on the results. Decreasing the amount of READASE reduces the amount of product.

The results show that labeled product was produced in reactions containing probes and targets where the potential hybrid would contain no mismatched bases between the probe and target in the region of the last few bases of the 3' end of the probe. The addition of additional targets to a single target probe combination did not prevent the production of specific labeled product. Finally, the presence of multiple matching primer target pairs were shown to all produce the expected labeled products.

Example 5: Effect of Shrimp Alkaline Phosphatase on  
the Detection of Label Incorporation

In the present example, a probe was pyrophosphorylyzed and then elongated by a single dideoxynucleotide as described earlier. The products were examined and demonstrated to have yielded the expected products only when the complementary probe and target were present together. In addition, unincorporated fluorescent nucleotides were de-phosphorylated using shrimp alkaline phosphatase to determine whether lower molecular weight bands were being obscured.

Eight reaction solutions were prepared to contain two tubes with 10  $\mu$ l of either target T1 or T2, such that there were 4 tubes with each target. Tubes were further prepared, in duplicate, such that two of the T2 tubes contained probe P2a, and two of the T2 tubes contained probe P1a. The remaining tubes were prepared, in duplicate, such that two of the T1 tubes contained probe P1a, and two of the T2 tubes contained probe P1b. The reaction was

permitted to proceed as described in previous examples. Reaction products were split into two tubes. One set was treated with Shrimp Alkaline Phosphatase (SAP, Promega, M8201) in order to modify the mobility of the excess labeled nucleotide by removal of the 5'-phosphate group.

Reaction products were loaded onto a gel, also as described in Example 2.

The results show reactions that contained matching (complementary) probes and target showed specific products plus the loss of non-specific staining at the bottom of the gel. No new lower molecular weight bands were observed. A non-complementary probe:target pair did not have any specific products.

Example 6: Specific Detection of a Leiden Mutation  
in a Human Nucleic Acid Sample

In the present example, a labeled probe annealed to a target was pyrophosphorylyzed and then elongated by a single dideoxynucleotide as discussed previously. The products were examined and demonstrated to have incorporated the expected nucleotide.

Oligonucleotide primer FVI (SEQ ID NO:18) and oligonucleotide primer FVI' (SEQ ID NO:19) were designed to amplify a region of the Factor V (five) gene from residual anonymous DNA from either a wild-type individual, an individual with a Leiden-type mutation, or from an individual that is heterozygous for the Leiden mutation.

Interrogation probe P<sub>FV</sub> (SEQ ID NO:20) was designed to hybridize with perfect homology to the wild-type sequence of the Factor V gene and to contain a fluorescein label incorporated into the 5'

end of the probe. Interrogation probe  $P_{LM}$  (SEQ ID NO:21) was designed to hybridize with perfect homology to the Leiden mutation sequence for the same gene and to contain a rhodamine label incorporated into the 5' end of the probe.

Primer FVI	BtnTEG-ACCCACAGAAAATGATGCCCG	SEQ ID NO:18
Primer FVI'	P-TTAAGTGCAAAAAGACAAGTAGCTTGTATT CTATAGTGTCACCTAAATC	SEQ ID NO:19
$P_{FV}$	F-CTTGAAGGACAAAATACCTGTATTCCTCG	SEQ ID NO:20
$P_{LM}$	TMR-CTTGAAGGACAAAATACCTGTATTCCTTG	SEQ ID NO:21

A mix of probes  $P_{FV}$  and  $P_{LM}$  was added to each of three reaction tubes containing either of three PCR products serving as targets, for the pyrophosphorolysis reaction. Master Mix for this reaction was added and the reaction allowed to proceed. Five  $\mu$ l of STR loading buffer (Promega Corp., DV4331) was added to the sample, and 5  $\mu$ l of this was run on non-denaturing 20%polyacrylamide gel. The gel was scanned using a Hitachi FMBIO. The first scan was at 505 nanometers, illuminating the fluorescein label and the second scan was at 585 nanometers, illuminating the rhodamine label.

Results of this study indicate that the wild-type and the heterozygote target show products under the 505 nanometer scan, but no products were visible in the lane containing only mutant target. Likewise, the mutant and the heterozygote target showed products under the 585 nanometer scan, but no products were visible if only wild-type target was present. Both scans confirmed the presence of a

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fluorescently labeled probe. These results indicate that it is possible to measure more than one target in a single reaction, and that using differently labeled probes increases the number of interrogations possible in a single reaction.

Example 7: Simplification of the Pattern of the  
Products of the Technology

In the present example, oligonucleotides complementary to the probes (designated shifters) are used to alter (shift) the mobility of the products produced using the technology such that they occupy less of the resolution area of non-denaturing gels. Such a reduction in the space occupied by any one set of products on the gel should allow a larger number of products to be individually visualized on one gel, thus increasing the level of multiplexed analysis that can be analyzed at one time. The shifters are added after the reaction has been terminated by addition of the commonly used metal chelator EDTA to a concentration in excess of the magnesium in the reaction. This prevents the shifters from acting as a template. The shifters are added in a molar excess of the total concentration of primer added to the reaction to allow all of the primer (both reacted and unreacted) to hybridize to the added oligonucleotide. In addition, the terminated reaction with the shifter added is heated to disrupt any primer-template hybrids in the solution and then cooled to allow the primer to hybridize to the shifter.

Probes P1a and P2a and Targets T2 and T1 are the same as in Example 1. Each probe (10  $\mu$ l) was mixed 10:200 in Nanopure™ (Millipore Corp.) water and

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MOPS-A to obtain a final concentration of 100 mM MOPS, 10mM MgCl<sub>2</sub>.

Probes and targets were prepared as earlier described. In addition two new oligonucleotides designated shifter oligonucleotides, S1,2a (SEQ ID NO:22) and S1,2b (SEQ ID NO:23) were designed to hybridize with probes P2a and P1a. The shifters are identical except that shifter S1,2a is 6 nucleotides longer than shifter S1,2b. Shifter oligos were prepared in EDTA-containing buffer (0.1mM EDTA, final concentration). A small amount of shifter oligo was also prepared in EDTA-free buffer.

S1,2a	HO-ATAGCGAAGAGGCCCGACCGATCGCC	SEQ ID NO:22
S1,2b	HO-ATAGCGAAGAGGCCCGACCG	SEQ ID NO:23

Two tubes were prepared to contain either 20  $\mu$ l of probe P2a and 20  $\mu$ l of its matching target, T2, or 20  $\mu$ l of probe P1a and 20  $\mu$ l of its matching target, T1. Master mix was prepared as in Example 1. 20  $\mu$ l of Master Mix was added to each of the two tubes, they were incubated at 55°C for one hour, and then stored at minus 20°C.

Ten tubes were prepared such that 5 each contained 4  $\mu$ l of each probe/target combination. To each of 4 of the probe/target combinations, 1  $\mu$ l of one of the shifter oligos was added, such that the 4 tubes each contained one probe, its matching target and one of the shifters. The concentration of shifters must be such that it is in Molar excess with respect to the interrogation probes to ensure that all the labeled probe is uniformly shifted. These were heated at 95°C for 2 minutes, then permitted to cool to room temperature. The heating step was only

necessary because the samples had been earlier frozen.

Four of the remaining tubes were prepared as above, except that after cooling to room temperature, the samples were precipitated using 5  $\mu$ l of 2M ammonium acetate (pH 4.6) and 90  $\mu$ l of 100% ethanol, then incubated at minus 20°C for about 20 minutes. The last two tubes were prepared with the shifter S1,2b, which had been prepared in EDTA-free buffer, and precipitated as above. Precipitated samples were resuspended. Gels were prepared as described in Example 1.

The results show that without the addition of shifters, multiple products are resolved in the gel for each reaction. In the samples treated with shifters as described above, only one major product is seen on the gel at a mobility dependent upon the length of the shifter added. Thus shifters can alter the mobility of the reaction products on the gel to a new location dependent upon the length of the shifter used and that they can be used to reduce the number of separate products species seen, thus increasing the number of different interrogation products that can be separately observed in one lane of a gel. In particular, these results specifically show that using shifters, one can achieve a product homogeneity in cases where two products are formed due to closely-spaced identical nucleotides (for example a "C" at the 3' terminus and at the minus 4 position). These results also indicate that EtOH precipitation may help to reduce the amount of free label present in a sample relative to the amount of product seen, and that EDTA does not seem to affect the labeled products seen from the reaction.

## Adrenal Hyperplasia gene in Humans

This example demonstrates that the technology can be used for determining the genotype of DNA segments that are associated with the human genetic disease known as Congenital Adrenal Hyperplasia (CAH). In addition, it was used to explore the effect of target concentration and NaOH concentration used to dilute the target on the amount of product made. Finally, a time course following the production of specific labeled primer products from probes designed to interrogate alleles associated with congenital adrenal hyperplasia was performed. The probes were designed to hybridize with total complementarity to one of four targets, all targets being different fragments of the same gene, the CAH gene (associated with congenital adrenal hyperplasia). The probe:target pairs are: P6-T6, P4-T4, P5-T5, P7-T7. The probes were pyrophosphorylyzed and then elongated by a single dideoxynucleotide as before. The products were examined and demonstrated to yield the expected products with high specificity. In addition, the data indicate that slightly changing the reaction pH by decreasing the concentration of the sodium hydroxide used to generate the working target solution did not affect the specificity of the reaction. Decreasing the amount of target in the reactions was shown to decrease the amount of labeled primer produced. The time course performed indicated that produce accumulation continued over the entire 60 minutes of the reaction incubation.

P4	HO-CACCTCCAGCCCCCAAC	SEQ ID NO:24
P5	HO-CACCTCCAGCCCCCACC	SEQ ID NO:25
P6	HO-CCCTCCAGCCCCCAGC	SEQ ID NO:26
P7	HO-TCTCTCCTCACCTGCAGCATCAAC	SEQ ID NO:27
T4	HO-AGGAGTTGGGGGCTGGAGGTGGGAA	SEQ ID NO:28
T5	HO-AGGAGGTGGGGGCTGGAGGTGGGAA	SEQ ID NO:29
T6	HO-AGGAGCTGGGGGCTGGAGGTGGGAA	SEQ ID NO:30
T7	HO-GTAACAGTTGATGCTGCAGGTGAGGAGAGAGAA	SEQ ID NO:31

Target oligonucleotides were prepared with Nanopure™ (Millipore Corp.) water to a concentration of approximately 1 mg/ml. Working target solutions were made by mixing target oligo stock solution (5 µl), water (10 µl), and 2X denaturation solution (60mM NaOH) to a final concentration of 25ng/µl in 0.03 N NaOH denaturation solution, as previously described. Target T4 was further prepared to provide the following: T4-1 was at 25ng/µl in 0.015 N NaOH; T4-2 was at 25ng/µl in 0.006 N NaOH; and T4-3 was at 5ng/µl in 0.03N NaOH.

The probe oligonucleotides were diluted in Nanopure™ (Millipore Corp.) water to a final concentration of 1 mg/ml. Working probe solutions were made by mixing probe, water and 2X MOPS (as previously described) to a final concentration of 50 µg/ml in MOPS-A (100 mM MOPS, 10mM MgCl<sub>2</sub>, pH 6.1).

Four tubes were prepared to contain 10 µl of each probe and 10 µl of its matching target. Three tubes were prepared with 10 µl of probe P4 and 10 µl of one of the targets T4-1, T4-2 or T4-3. Two tubes were prepared with 25 µl of P4 and either 25 or 5 µl of T4, the balance of volume in the tube given 5 µl of T4 being made up by Nanopure™ (Millipore Corp.)

water. These two tubes are labeled 8 and 9. Lastly, eight tubes were prepared containing only 1 $\mu$ l of 0.5M EDTA. These were labeled "8T0" through "8T3", and "9T0" through "9T3".

Master Mix was prepared as previously described in Example 1. 10  $\mu$ l of Master Mix was added to the first 7 tubes, and tubes were placed at 55°C.

25  $\mu$ l of Master Mix was added to tubes 8 and 9 and mixed. Immediately, 10  $\mu$ l from tubes 8 and 9 were transferred to tubes 8T0 or 9T0; ( $t_0$  = zero minutes at 55C), and tubes 8 and 9 were placed at 55°C. After 15 minutes of incubation at 55C, another 10  $\mu$ l was removed from tubes 8 and 9 and transferred to tubes 8T1 and 9T1 ( $t_1$  = 15 minutes), and incubation continues. After 30 minutes of incubation at 55C, a third 10 $\mu$ l aliquot was removed from tubes 8 and 9 and transferred to tubes 8T2 and 9T2 ( $t_2$  = 30 minutes) and incubation continues. At 45 minutes a fourth 10 $\mu$ l aliquot was removed from the reaction tubes 8 and 9 and transferred to tubes marked 8T3 and 9T3 ( $t_3$  = 45 minutes) and incubation continued. At 60 minutes, all tubes are removed from the heater and placed at minus 20°C.

Gel electrophoresis was carried out as earlier described. The results show that labeled products were produced in the reactions containing matching probe:target pairs and indicate that there is no apparent effect on the amount of product made as a result of the slight pH change produced in the reaction by altering the concentration of sodium hydroxide used in the target solution. In addition, the results show that reducing the level of target reduces the amount of product produced. Finally

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examination of the samples taken at 0, 15, 30, 45 and 60 minutes indicates that product continued to accumulate over the entire time period of the incubation, so as the reaction time is extended, the amount of product formed can be increased.

Example 9: Effect of the Concentration of  
Probe or Target on the Assay

In the present example, the concentration of probe or target present was varied in the reaction and they were held at constant temperature. The products were examined and demonstrated to have yielded the expected products, with increasing amount of target or probe having a positive effect on the visibility of reaction products on a gel.

A "1X" probe solution, designated P3b in the table below, was produced by diluting 5µl of a 1mg/ml oligonucleotide stock of P3b (Sequence ID NO:11) with 45µl of water and 50µl of MOPS A (see Example 1). Solution P3bx2 was prepared by dilution 10µl of the 1mg/µl P3b stock with 40µl of water and 50µl of MOPS A. Solution P3bx4 was prepared by diluting 20µl of the 1mg/ml solution of P3b with 30µl of water and 50µl of MOPS A.

Template (target) solution T was prepared by diluting 5µl of stock oligonucleotide T3 (1mg/ml) (sequence ID NO:17) with 95µl of water and 100µl of denaturation solution (60mM sodium hydroxide). Template solution T3x2 was prepared as was solution T except that 10µl of stock oligonucleotide was used and the water reduced to 90µl. Template solution T3x4 was prepared as was

solution T except that 20 $\mu$ l of the stock oligonucleotide was used and the water reduced to 80 $\mu$ l.

Master mix was assembled as in Example 1 and the reactions shown in the table below were assembled and incubated at 55°C for 60 minutes.

Tube	Probe ( $\mu$ l)	Target ( $\mu$ l)	MM ( $\mu$ l)	Temp (°C)	Time (min)
2	10 of P3bx2	10 of T3x02	10	55	60
5	10 of P3b	10 of T3x02	10	55	60
6	10 of P3bx2	10 of T3x02	10	55	60
7	10 of P3bx4	10 of 3T3x02	10	55	60
1	10 of P3bx2	10 of T3	10	55	60
3	10 of P3bx2	10 of T3x4	10	55	60
4	10 of P3bx2	10 of T3x1	10	55	60

The products produced were visualized by gel electrophoresis and fluorometry as described in Example 1. The results indicate that the concentration of either primer or target does not affect the specificity of the technology, but may affect amounts of products seen on the gel. In this case, increasing concentration of the primer may increase the signal strength of the product, and decreasing concentration of the target may decrease signal strength. Optimization of these concentrations may depend upon the nature of the

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### Example 10: Effect of Thermal Cycling

Tubes were prepared to contain 50  $\mu$ l of each target and each buffer, such that there were 4 tubes, labeled 1 through 4, with each target dilution present with each of the two probes. Two additional sets of tubes labeled 1 through 4 were also prepared.

The results show that low target concentrations produce less labeled product formation under any of the three reaction conditions. The

Example 11: Thermal Cycling Equalizes the Length Effect of Short and Long Probes

In the present example, probes of various sizes were annealed to a target and permitted to undergo pyrophosphorylysis either under constant temperature conditions or thermal cycling conditions as discussed before.

A set of six tubes was prepared in duplicate, totaling twelve tubes. Target solution

T3A was prepared as in Example 10. Probes P3a, P3c, P3d, P3e, P3f and P3g were also prepared as earlier described, in a MOPS solution (as described in Example 3).

Tubes were prepared in duplicate such that each tube contained 10  $\mu$ l of target solution, and two tubes each contained 10  $\mu$ l of one of the probes.

Master mix was prepared as earlier described, and 10 $\mu$ l was added to each of the 12 tubes, above. One set of six tubes was incubated at 55°C for one hour. The second set of tubes was incubated at 55°C for 4 minutes and 45 seconds followed by 14 seconds at 85°C, and cycled for one hour.

The results show that generally, for the longer probes, product formation is enhanced when the reaction is thermally cycled. Optimization of thermal cycling does not require undue experimentation.

Example 12: Conditions For Detection May Vary  
Depending upon the Nucleotide  
Composition of the Assayed  
Nucleic Acid Hybrid

This example shows that, although fluorescent oligonucleotide incorporation is specific, conditions for detection of the nucleic acid hybrid via pyrophosphorolysis and incorporation of nucleotide methods varies predicted melting temperature of the probe/target nucleic acid hybrid and can be ascertained without undue experimentation. Two probes were designed to hybridize to a target oligo with a preferred melting temperature, both targets being different fragments of the same gene,

the CAH gene (associated with congenital adrenal hyperplasia). The probe target pairs are: P8-T8 and P9-T9. The probes were pyrophosphorylyzed and then a single dideoxynucleotide was incorporated into the probe, the reaction taking place either at constant temperature or in a thermal cycling protocol as discussed previously. The products were examined and demonstrated to yield the expected products with high specificity.

P8	HO-TCCTCCGGATCAATCCCCAGATTC AGCAGCGACTGC	SEQ ID NO:32
P9	HO-ACTCCTCCGGATCAATCCCCAGAT TCAGCAGCGACTGT	SEQ ID NO:33
T8	HO-ACCTGCAGTCGCTGCTGAATCTGG GGAATGATCGGGAGGAGTCCTGC	SEQ ID NO:34
T9	HO-ACCTACAGTCGCTGCTGAATCTGG GGAATGATCGGGAGGAGTCCTGC	SEQ ID NO:35

Targets and probes were prepared in solutions as described earlier. Four tubes were prepared in duplicate such that there are two sets of tubes, with two each containing a probe and target pair.

Master Mix was prepared as described earlier, except that a fluorescent guanosine is the labeled nucleotide. Ten  $\mu$ l of the Master Mix (G) was added to each tube, and one set of tubes was incubated at 55°C for one hour, and the second set of tubes was incubated as in Example 11.

The results indicate that in the absence of the appropriate probe:target pair, there is no product formation. The results also indicate that some optimizing may be necessary for particular

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probes. This finding is a reasonable expectation, because nucleotide content directly influences hybridization optimization. For example, mismatched bases could be deliberately incorporated into longer probes to allow for a desired melting temperature of the hybrid pair.

Example 13: Conditions For Detection of the  
Assayed Nucleic Acid Hybrid II

The present example is very similar to Example 12, but uses different probe:target pairs. Two probes were designed to hybridize to a target oligo with a preferred melting temperature, both targets being different fragments of the same gene, the CAH gene (associated with congenital adrenal hyperplasia). The probe target pairs are: P10-T10 and P11-T11. The probes were pyrophosphorylyzed and then elongated by a single dideoxynucleotide, the reaction taking place either at constant temperature or in a thermal cycling protocol as discussed before. The products were examined and demonstrated to yield the expected products with high specificity.

P10	HO-GCCCTTGGTCAATCCCACGATCGCCGA GGTGTGCGCCTGCGG	SEQ ID NO:36
P11	HO-GCCCTTGGTCAATCCCACGATCGCCGA GGTGTGCGCCTGTGG	SEQ ID NO:37
T10	HO-AACGGGCCGAGGCGCAGCACCTCGGC GATGGTGGCATTGAGCAAGGGCA	SEQ ID NO:38
T11	HO-AACGGGCCACAGGCGCAGCACCTCGGC GATGGTGGCATTGAGCAAGGGCA	SEQ ID NO:39

Targets and probes were prepared in solutions as described previously. Four tubes were prepared in duplicate such that there were two sets

of tubes, with two each containing a probe and target pair.

A Master Mix was prepared as described before, with a fluorescent guanosine as the labeled nucleotide. Ten  $\mu$ l of the Master Mix (G) is added to each tube, and one set of tubes was incubated at 55°C for one hour, and the second set of tubes was incubated as in Example 11.

The results show that in the absence of the appropriate probe:target pair, there is no product formation. The results also confirm that some optimizing may be necessary as described earlier.

Example 14: Nucleic Acid Hybrid Detection Using a  
Solid Support for One Hybrid Strand

This example demonstrates one embodiment of a nucleic acid hybrid detection method using a solid support to grasp one strand of the nucleic acid hybrid. A solid support, a TETRALINK resin (Promega catalog number B259), was prepared such that it was bound with oligo probe number P12. This probe also contains a biotinylated moiety (Btn-) at the 5' end. Two target probes were prepared such that one would match with perfect homology to probe P12 (Target T12) and the other T12\*.

P12	Btn-CCTCTTCGCTATTACTTCGCCATTTGACGTT GGAG	SEQ ID NO:40
T12	HO-GAGTTCT <u>CC</u> CAACGTCAAATGGCGAACATTA	SEQ ID NO:41
T12*	HO-GAGTTCTG <u>CC</u> CAACGTCAAATGGCGAACATTA	SEQ ID NO:42

The resin was prepared according to manufacturer's instructions, in brief, 200  $\mu$ l of

resin was placed on a BIO-RAD poly-prep column and washed with 10 ml of nuclease-free water. The column was then equilibrated with 10 ml of 50 mM Tris-HCL pH 7.5/0.1% Tween 20. After most of the equilibration buffer had passed through the column, 100 µl of probe P12 at 1 mg/ml was placed on the column. The resin was mixed briefly with a Pasteur pipet and permitted to incubate for 15 minutes at room temperature. The column was then washed with 10 ml of 10 mM Tris-HCL (w/o Tween), followed by 10 ml of nuclease-free water. Oligo-resin was then transferred to a microfuge tube.

Two hundred microliter of resin was mixed with 200 µl of neutralization solution (200 mM MOPS, 6.15; 20 mM  $MgCl_2$ ).

Targets were prepared such that 50 µl of T12 and T12\* (at 1 mg/ml) were added to 50 µl of nuclease free water, in duplicate, such that there were two tubes of each target.

Master Mix was prepared with 190 µl of NANOPURE water, 5 µl of MOPS-A (200 mM MOPS, 20 mM  $MgCl_2$ , pH 6.1), 8 µl of  $NaPP_i$  (40 mM), 5 µl F-12-ddCTP, and 2µl of READASE™ Polymerase (Promega EAP#2) or 2 µl of nuclease free water.

Fifty microliters of labeled resin were added to each of the four tubes containing target. To one set of tubes (one of each target) was added 100 µl of the Master Mix, and to the second set of tubes was added 100 µl of control Master Mix (no enzyme). All tubes were permitted to incubate at 55°C for one hour.

After incubation, contents of each tube were placed into separate fresh poly-prep columns

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From the foregoing, it will be observed that numerous modifications and variations can be effected without departing from the true spirit and scope of the present invention. It is to be understood that no limitation with respect to the specific examples presented is intended or should be inferred. The disclosure is intended to cover by the appended claims modifications as fall within the scope of the claims.